

AD _____

Award Number: DAMD17-98-1-8601

TITLE: Therapeutic Resistance of Prostate Cancer Mediated by
Cadherin T6

PRINCIPAL INVESTIGATOR: Ralph Buttyan, Ph.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

20001018 036

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1999	3. REPORT TYPE AND DATES COVERED Annual (1 Oct 98 - 30 Sep 99)	
4. TITLE AND SUBTITLE Therapeutic Resistance of Prostate Cancer Mediated by Cadherin T6			5. FUNDING NUMBERS DAMD17-98-1-8601	
6. AUTHOR(S) Ralph Buttyan, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Columbia University New York, New York 10032 E-Mail: rb4@Columbia.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) In order to better understand the molecular basis for the development of therapeutic resistance by prostate cancer, we have undertaken experimentation in which we genetically compared therapy resistant prostate cancer cells to therapy sensitive prostate cancer cells. As a result of this comparison, we have identified a novel gene product, previously referred to as T6 (now referred to as Protocadherin-PC) that is highly upregulated when prostate cancer cells are selected for resistance to apoptotic agents in vitro and when these cells are selected for hormonal resistance in vivo. This progress report describes our success in characterizing the human Protocadherin-PC gene and gene product and in describing the probable mechanism through which this protein increases therapeutic resistance of prostate cancer cells. As a result of these studies, we believe that prostate cancer progresses to the state of therapeutic resistance in a mechanistically similar manner to that of human colon cancer and melanoma.				
14. SUBJECT TERMS Prostate Cancer			15. NUMBER OF PAGES 24	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4 - 9
Key Research Accomplishments.....	9 - 10
Reportable Outcomes.....	10
Conclusions.....	10 - 11
References.....	11
Appendices.....	12-24

INTRODUCTION

Advanced human prostate cancer rapidly develops resistance to the hormonal and other therapeutic agents used to treat it. In order to better understand the genetic changes that accompany the development of therapeutic resistance by prostate cancer cells, we have developed human prostate cancer cell lines with a generic resistance to agents that normally induce apoptosis and we have genetically compared these cell lines to the parental, therapeutic-sensitive cells from which they were derived. These experiments enabled us to identify a partial cDNA sequence, originally referred to as T6, that is expressed in the therapeutically resistant cell lines but not in the parental prostate cancer cells. Preliminary characterization of this gene product showed that it was a new member of the cadherin gene family, a family of gene products that are thought to be important for intracellular adhesion as well as involved in cellular signaling processes. Our application proposed the full characterization of the T6 gene product by complete sequencing of the cDNA, identification of T6 gene structure and the development of antibodies against the protein encoded by the T6 cDNA. These goals have been totally accomplished during the first year of the project period and this work has revealed the unique and unusual nature of this apoptosis-regulating gene product that has now been renamed "Protocadherin-PC". In addition we have been able to identify a putative mechanism through which the protocadherin-PC molecule might alter prostate cancer cell sensitivity to therapeutic agents ; apparently this molecule promotes signaling through the β -catenin pathway and our findings indicate that prostate cancer cells can progress to hormone resistance through a mechanism that is similar to human colon cancers and melanomas. We have also developed the means to survey human prostate cancer specimens for expression of this gene product and our preliminary survey indicates that protocadherin-PC is highly expression in actual hormone-resistant human prostate tumors and that the expression of protocadherin-PC in these tumors correlates with abnormalities in β -catenin localization. Finally, we have identified the chromosomal location of the gene encoding protocadherin-PC and, interestingly, it is on the Y-chromosome, a chromosome that frequently demonstrates anomalies in prostate cancer cells.

Body

Task 1. *To fully characterize the human gene encoding T6 as well as its mRNA and protein products.*

This project originated from studies in which we derived apoptosis resistant variants of the LNCaP cell line by sequential repeated exposure of parental LNCaP cells to one of two different agents that induce apoptosis of these cells (phorbol esters or serum starvation) followed by expansion of the surviving cell populations. The two resistant cell lines referred to as LNCaP-TR (for TPA resistant) and LNCaP-SSR (for serum starvation resistant) were shown to be resistant to the alternate agent that was not used in their selection and they were also shown to be hormone resistant by testing for their ability to form tumors in castrated male immunodeficient mice following xenografting. Thus they were found to be generically "apoptosis-resistant" when compared to the parental LNCaP cells from which they were derived. A subtractive hybridization-PCR technique was then used to comparatively screen for genetic differences between LNCaP-TR and parental LNCaP cells and this screening technique enabled us to identify a fragment of cDNA, that we initially referred to as T6, that was preferentially expressed in the LNCaP-TR cells. Hybridization of this cDNA fragment to a Northern blot demonstrated that an approximate 4.5 kb mRNA homologous to the probe is highly expressed in both the LNCaP-TR as well as the -SSR cells and also demonstrated that this same transcript is highly upregulated in the parental LNCaP cells following prolonged exposure to a testosterone-deficient medium. An RNase protection

assay showed that this message is highly induced in LNCaP tumor xenografts when they are selected for their ability to grow in a testosterone-deficient environment following castration of the host mouse.

In this first year of the project, sequence information from the T6 tag fragment was used to generate PCR primer sets for 5' and 3' RACE procedures to obtain the complete sequence of the homologous cDNA. These procedures resulted in the cloning of complete 5' and 3' cDNA fragments, each of which were sequenced by standard dideoxynucleotide techniques. Analysis of the complete sequence for the 4.8 kb cDNA (Figure 1) showed the presence of one long open reading frame of 2,868 bases that would translate to a polypeptide with 956 amino acids with a predicted molecular weight of approximately 105.9 kd. The translation product has 7 sequential regions that are characteristic of cadherin box domain sequences near the N-terminus. This cDNA thus identifies a new member of the cadherin superfamily of gene products and we have named it protocadherin-PC based upon the multiple nature of the cadherin box domains (more than 5) which is a proposed characteristic of the cadherin subfamily referred to as protocadherins (1) and because computerized (Fasta) searches of Genpept hit several protocadherins with E less than 0.001, but not classical cadherins with that low an E value. Protocadherin-PC also contains a serine-rich region near the C-terminal with partial homology to the β -catenin-binding site found on several classical cadherins (2). Another unusual aspect of the translation product of T6 is its lack of a signal sequence. This deficiency makes it unlikely that the protocadherin-PC polypeptide would get through the endoplasmic reticulum and into the cell membrane, like most other members of the cadherin gene family.

A 15 amino acid peptide corresponding to residues 829-843 of the predicted protocadherin-PC polypeptide was synthesized and used to repeatedly immunize rabbits. Serum from immunized rabbits was used to probe a Western blot that contained proteins extracted from parental LNCaP cells and from the -TR and -SR variants. This serum specifically recognized a protein of approximately 110 kd on Western blots that was present in abundance in the apoptosis-resistant LNCaP variants but was only present in trace amounts in the parental LNCaP cells (Figure 2). A similar sized protein recognized by the antiserum was found to be abundantly present in extracts made from LNCaP tumor xenografts obtained after castration of the host mouse, but not before (Not shown). Cellular fractionation showed that this 110 kd antigen was cytoplasmically localized (Figure 3) and together, these observations support the likely identify of the 110 kd band on Western blots as the protein product encoded by the protocadherin-PC transcript.

In order to confirm the potential for β -catenin binding by the protocadherin PC protein, we directly immunoprecipitated protocadherin PC from cytosolic extracts of the LNCaP cell variants and then tested these immunoprecipitates by means of Western blotting to determine whether β -catenin protein was co-precipitated. The results of the Western blot (Figure 4) showed that the 94 kd β -catenin protein was present in the immunoprecipitates of the -TR and -SSR cells but not from the parental LNCaP cell that express low levels of protocadherin PC mRNA.

Cadherin-family proteins are usually found in the outer membrane where they function to mediate intracellular adhesion between epithelial cells (3). The cytoplasmic domains of the classical cadherins are also known to directly or indirectly bind to several different proteins referred to as catenins. These proteins can influence the structural integrity of a cell (α -catenin anchors actin filaments to the cell membrane) or participate in the regulation of gene expression through the Wnt/Wingless signaling pathway (β -catenin is a transcriptional regulatory protein that complexes with the Lef-1/Tcf family of

transcription factor proteins) (3). In normal epithelium, β -catenin is tightly retained at its cadherin binding site on the membrane. Any free cytoplasmic β -catenin is rapidly degraded by a complex reaction controlled by the Adenomatous Polyposis Coli (APC) protein (4). In colon and some other cancers, mutations in the APC protein or in β -catenin can stabilize β -catenin so that it accumulates in the cytoplasm and nucleus where it stimulates the expression of growth and survival proteins of these cells (5). Given this complex relationship and the potential for disruption of such a relationship by a new cytoplasmically-localized cadherin-related protein with β -catenin binding capacity, we surveyed our cell lines for the expression and compartmentalization of the cadherin-binding proteins, α - and β -catenin.

Parental LNCaP cells and the -TR and -SSR derivatives were fractionated into membrane, cytoplasmic and nuclear fractions and the proteins in these fractions were electrophoresed on an SDS-polyacrylamide gel and then transferred to a nitrocellulose filter. The Western blots were probed with antibody against E-cadherin, or against α - or β -catenin. As shown in Figure 5 (Top Panel), E-cadherin in each of the cell derivatives was totally restricted to the membrane fractions. Comparison of E-cadherin levels between the various lines did appear to show a reduction in this cellular adhesion and metastasis-suppressing protein in the apoptosis-resistant variants, -SSR and -TR. α -catenin, a structural protein, appeared to be equally expressed amongst the different cell lines and equally distributed between the membrane and the cytosolic fraction of each cell line (Figure 5, Center Panel). In contrast, although overall β -catenin levels were found to be similar in the 3 variant cell lines, the distribution of β -catenin protein was found to be remarkably different amongst the lines. In the parental LNCaP cells, β -catenin was exclusively retained in the cell membrane fraction, whereas in the -SSR line, most of the β -catenin is associated with the cytoplasmic fraction and in -TR cells the nuclear fraction (Figure 5, Bottom Panel). This finding demonstrates that enhanced expression of the protocadherin-PC in the LNCaP-TR and -SSR cell lines is also associated with a remarkable change in the intracellular distribution of β -catenin, a protein which has the distinct potential of enhancing cellular survival when its nuclear transcriptional activity is facilitated.

The altered intracellular distribution of β -catenin that we detected in the apoptosis-resistant cell lines was also found to be associated with a coordinate increase in endogenous transcriptional activity from the Lef-1/Tcf transcriptional response element in these cells. When the LNCaP cell variants were individually co-transfected with a luciferase reporter plasmid containing Lef-1/Tcf response elements (pTOP-Flash) (6, 7) and a β -galactosidase reporter plasmid promoted by a simple CMV promoter element, the ratio of luciferase/ β -galactase activity was found to be over 2-fold greater than parental LNCaP in the -SSR line and over 4-fold greater than LNCaP in -TR line (Figure 6a). Likewise, when we compared expression of luciferase reporter in these lines after transfection with pTOP-Flash (promoted by the optimal Lef-1/Tcf response element) or pFOP-Flash (luciferase reporter promoted by a mutated, inactive Lef-1/Tcf response element) (6, 7) we found similar results; i.e. a greater than 2-fold increased ratio of pTOP/pFOP luciferase activity in the -SSR cells and a greater than 2.5-fold increased ratio of pTOP/pFOP luciferase activity in the -TR cells (Figure 6b). These results demonstrate that endogenous transcriptional activity promoted by the Lef-1/Tcf response element is significantly greater in the protocadherin-PC expressing LNCaP lines, -TR and -SSR than in the parental, apoptosis-sensitive LNCaP line and support the concept that the β -catenin-regulated transcriptional pathway is more active in the -SSR and -TR lines that express protocadherin-PC.

In a search of genbank for homologous sequences to protocadherin-PC cDNA, we identified a human genomic entry that had sequences with virtual complete homology to protocadherin-PC. These sequences were present in a contiguous DNA fragment isolated from the human X-chromosome (Accession # ac004388). Comparison of the genomic sequence to the cDNA sequence of protocadherin-PC allowed us to map the putative intron/exon structure of the protocadherin-PC-like cDNA present in the X chromosomal fragment. This comparison defined a potential protocadherin-PC-like gene product with 7 distinct exons that differed from protocadherin-PC cDNA only within the fifth exon, wherein an extra contiguous 13 bp sequence is present that is not found in the sequence of the protocadherin-PC cDNA isolated from the LNCaP cell derivatives. Computer analysis of the potential open reading frames encoded by the protocadherin-PC-like gene structure present on the X-chromosomal fragment (with the 13 bp addition) showed that the putative transcript from this gene contained one long open reading frame that would also translate into a cadherin-related protein. However, although this polypeptide retains complete homology to the amino acid structure of the prostate cancer protocadherin-PC throughout most of its carboxy terminal domain, it differs significantly from the polypeptide encoded by the protocadherin-PC transcript cloned from prostate cancer cells at its N-terminal domain, having a signal sequence that would enable it to be inserted into membranes. This is caused by a frame shift introduced by the extra 13 bp that affects the amino acid coding potential of the 5' region of the transcript and enables the use of an alternate methionine start further upstream of the first potential methionine encoded by the protocadherin-PC transcript that lacks the 13 bp sequence (Figure 7a).

A more recent resurvey of genbank revealed a new entry (human genomic DNA) that contains the complete truncated (cytoplasmic) form of protocadherin-PC on a contiguous human DNA fragment derived from the Y-chromosome (accession #ac010722). Thus the 2 different forms of protocadherin-PC appear to be near-duplicate versions of the same gene, located on different sex chromosomes. The truncated (cytoplasmic) form, encoded on the human Y chromosome, is the form that is expressed in abundance in the apoptosis-resistant prostate cancer cell derivatives and in hormone-resistant human prostate tumors. As well, this is likely to be the form of protocadherin-PC that has the capability of activating the β -catenin signaling pathway.

Task 2. *To determine whether genetic manipulation of cultured prostate cancer cells so that they overexpress T6 (Now Protocadherin-PC) cDNA diminishes their responsiveness to apoptotic agents in vitro and in vivo.*

We are still in the process of completing this task. Although we were able to derive the complete sequence of the Protocadherin-PC transcript based on 5' and 3' RACE cloning techniques, it proved more difficult to PCR the complete 4.8 kb cDNA, however this has now been accomplished. We are presently installing the cDNA fragment into several different expression vectors and will be completing this task during the remainder of the project period.

Task 3. *To determine whether T6 (now Protocadherin-PC) expression influences the metastatic phenotype of prostate cancer cells.*

Completion of this task requires protocadherin-PC-transformed LNCaP cells which is in process of being completed at this time.

Task 4. *To evaluate clinical specimens of human prostate cancer to determine whether T6 (Now Protocadherin-PC) expression correlates with therapeutic resistance of clinically aggressive prostate cancer.*

In order to evaluate whether protocadherin-PC might be involved in the natural progression of human prostate cancers, we utilized a semi-quantitative RT-PCR assay (8) to evaluate expression levels of protocadherin-PC mRNA in RNAs extracted from 64 different human specimens consisting of normal or diseased (benign and malignant) human prostates. The assay compares the relative amplification efficiency of a small internal fragment of protocadherin-PC (260 bp) cDNA to the amplification of a fragment of a constitutive transcription factor cDNA (TBP). This assay, when applied to cDNAs from the LNCaP cell variants, confirmed the significant upregulation of protocadherin-PC mRNA expression in the -TR and -SSR variants and showed that cultured normal human prostate cells (epithelium or stroma) lacked protocadherin-PC expression (Figure 8a). When the assay was applied to RNAs extracted from human prostate tissues, the results (Figure 8b) showed that there was low-level expression of protocadherin-PC-related mRNA in all normal prostate tissues, regardless of the anatomical zone of the prostate from which they were derived (peripheral, central or transitional zone), measured as a mean relative expression of 0.302 ± 0.169 ; 0.411 ± 0.119 ; or 0.231 ± 0.134 , respectively. A similar low level expression was maintained in specimens of diseased prostate tissues consisting of benign prostatic hyperplasia (BPH) or untreated (localized) prostate cancers (mean relative expression 0.287 ± 0.131 in BPH and 0.196 ± 0.204 in cancer). However, in contrast, tumors obtained from prostate cancer patients that were experiencing disease progression in spite of hormonal therapy (hormonal failure) were found to have a mean expression of protocadherin-PC mRNA that was significantly greater than any of the other types of tissues or tumors (mean relative expression = 1.031 ± 0.896 ; $p < 0.05$). This difference in protocadherin-PC mRNA expression was also found when tissue sections containing untreated prostate cancer or hormone resistant prostate cancer were compared following *in situ* hybridization for protocadherin-PC (not shown here). Significantly more intense hybridization was observed in the cancerous cells of all tumors obtained from patients with 6 months or more of hormonal therapy than was found in cancer cells in tumors from untreated patients. This data supports the results of our semi-quantitative RT-PCR assay and suggests that prostate cancer cells that survive after prolonged hormonal deprivation therapy of prostate cancer patients express much higher levels of protocadherin-PC-related mRNA when compared to prostate cancer cells that are therapeutically-naïve. Since the normal prostate tissues were found to express a low level of protocadherin-PC-related transcript, we also surveyed several other normal human tissues (brain, heart, liver, muscle, kidney, duodenum, placenta) using the same semi-quantitative RT-PCR assay. This survey showed that protocadherin-PC-related transcripts could also be detected as well in RNAs extracted from normal human brain (protocadherin-PC/TBP = 3.76) and placenta (protocadherin-PC/TBP = 2.87).

Using a primer set that would specifically amplify a small region within the putative fifth exon of protocadherin-PC (spanning the site containing the 13 bp cassette), we performed RT-PCR on cDNA of normal human brain or human placenta. The primers amplified only a single band of 130 bp from each of these tissues, that, when sequenced, proved to be the cDNA form of protocadherin-PC containing the 13 bp insertion that confers a membrane-binding potential on the translated protein. In contrast, when these primers were utilized to amplify cDNA extracted from the LNCaP cell variants or from normal human prostate or human prostate tumors, there were typically two amplification products, one at 130 bp and another at 117 bp (Figure 9). When the different sized amplification products were sequenced they proved to be the two different forms of protocadherin-PC, with or without the 13 bp insertion. Moreover,

it was clear from the abundance of the different forms of the RT-PCR products amplified from the prostate cancer cells and human prostate tissues/tumors that it is the expression of the truncated (cytoplasmic) form of protocadherin-PC mRNA that is increased in abundance when the LNCaP cells were selected for apoptosis-resistance or when primary human prostate cancers acquire hormone resistance (Figure 8b).

Key Research Accomplishments

- The complete cDNA sequence of Protocadherin-PC (Formerly T6) has been obtained
- The translation product of Protocadherin-PC has been described
- An polyclonal antibody was made that recognizes the Protocadherin-PC protein on Western blots
- The Protocadherin-PC protein was found to be localized within the prostate cancer cell cytoplasm
- The Protocadherin-PC protein was shown to functionally bind to β -catenin protein
- Apoptosis-resistant LNCaP cell variants that express Protocadherin-PC were found to have abnormal β -catenin localization (cytoplasmic and nuclear instead of membrane-bound)
- Apoptosis-resistant LNCaP cell variants that express Protocadherin-PC were found to have enhanced signaling through the β -catenin pathway
- The Protocadherin-PC gene was mapped to the human Y-chromosome
- A variant gene highly related to Protocadherin-PC was mapped to the X-chromosome (near Xq24)
- The X-chromosomal Protocadherin-PC-like gene was found to be membrane bound (in contrast to Protocadherin-PC which is cytoplasmically localized) and is expressed normally in human brain and placenta
- A survey of actual human prostate cancer specimens demonstrates that Protocadherin-PC expression is low in normal prostate, benign prostate hyperplasia and untreated prostate cancer but is significantly higher in hormone-resistant prostate tumors
- Coordinate analysis for expression of Protocadherin-PC and its homologue on the X-chromosome in prostate cancer specimens demonstrate that it is the truncated, cytoplasmic form of Protocadherin-PC (from the Y-chromosome) that is upregulated in hormone-resistant prostate cancer cells

- **Preliminary analysis of hormone-resistant human prostate tumors that express high levels of Protocadherin-PC (cytoplasmic form) demonstrate coordinate dysregulation of β -catenin protein (found predominantly in the cytoplasm and nucleus instead of on the membrane)**
- **The complete cDNA encoding Protocadherin-PC has been cloned and inserted into eukaryotic expression vectors in anticipation of producing LNCaP transformants that express the Protocadherin-PC protein**

Reportable Outcomes

Manuscripts – Chen, M.-W., de la Taille, A., Vacherot, F., Gil-Diez-de-Medina, S., Shen, R., Friedman, R.A., Kitajewski, J., Burchardt, M., Chopin, D.K. and Buttyan, R. (2000) The emergence of protocadherin-PC expression during the acquisition of apoptosis-resistance by prostate cancer cells. *Nature Medicine* (Submitted).

Patents, Licenses – None.

Degrees – None.

Cell Lines, Tissues or Serum Repositories – Development of two apoptosis-resistant prostate cancer cell derivatives of the LNCaP lineage, LNCaP-TR and LNCaP-SSR. Eukaryotic expression vectors with cDNA encoding human protocadherin-PC

Informatics – Sequence of Protocadherin-PC cDNA (Submitted to Genbank in conjunction with manuscript to *Nature Medicine*)

Funding Applied For – A Phase II application for completion of this project (PC001565 – *Protocadherin-PC and Therapeutic Resistance of Prostate Cancer*, R. Buttyan, P.I., submitted May 17, 2000)

Employment/Research Opportunities Applied For – None

Conclusions

This project has allowed the description of a unique and novel mechanism whereby prostate cancer cells can develop therapeutic resistance to hormonal-ablation as well as other therapeutic agents. Our experimentation has demonstrated that acquisition of therapeutic resistance by prostate cancer cells is accompanied by increased expression of a very unusual cadherin-family protein now named Protocadherin-PC. The product of the Protocadherin-PC gene, located on the human Y chromosome, lacks a signal sequence, and, as a result, its translation product is cytoplasmic instead of membrane bound as with other members of the cadherin gene family. Our preliminary experimentation suggests that Protocadherin-PC might stimulate apoptosis resistance of prostate cancer cells by activating signaling through the β -catenin pathway, a signaling pathway that is also known to be involved in the progression of human colon cancer and melanoma. Moreover, our survey of actual human prostate tumors demonstrates that expression of this cytoplasmic form of Protocadherin-PC is significantly correlated with the hormone-resistant phenotype and disturbances of the β -catenin signaling pathway. These results

suggest that Protocadherin-PC and the β -catenin signaling pathway might be important targets for the development of therapeutics against hormone-resistant prostate cancer.

References

1. Suzuki, S.T. Structural and functional diversity of cadherin superfamily: are new members of cadherin superfamily involved in signal transduction? *J. Cell Biochem.* **61**, 531-542 (1996).
2. Stappert J. & Kemler, R. A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes. Commun.* **2**, 319-327 (1994).
3. Aoki, M., Hecht, A., Kruse, U., Kemler, R. & Vogt, P.K. Nuclear endpoint of Wnt signaling: neoplastic transformation induced by transactivating lymphoid-enhancing factor 1. *Proc. Natl. Acad. Sci., U.S.A.* **96**, 139-144 (1999).
4. Moon, R.T. & Miller, J.R. The APC tumor suppressor protein in development and cancer. *Trends Genet.* **13**, 256-258 (1997).
5. Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. & Kinzler, K.W. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**, 1787-1790 (1997).
6. Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O. & Clevers, H. Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. *Mol. Cell. Biol.* **18**, 1248-1256 (1998).
7. Ishitani, T., Ninomiya-Tsuji, J., Nagai, S., Nishita, M., Meneghini, M., Barker, N., Waterman, M., Bowerman, B., Clevers, H., Shibuya, H. & Matsumoto, K. The TAK1-NLK-MAPK-related pathway antagonizes signaling between beta-catenin and transcription factor TCF. *Nature* **399**, 798-802 (1999).
8. Diez de Medina, S.G., Chopin, D., El Marjou, A., Delougee, A., LaRochelle, W.J., Hozneck, A., Abbou, C., Aaronson, S.A., Thiery, J.P. & Radvanyi, F. Decreased expression of keratinocyte growth factor receptor in a subset of human transitional cell bladder carcinomas. *Oncogene* **14**, 323-330 (1997).

Appendices

Figures 1-86 (Attached).

APPENDIX

Progress Report: DAMD17-98-1-8601

Therapeutic Resistance of Prostate Cancer Mediated by Cadherin T6

Figures 1 – 8b

Legends to Figures

Figure 1. Partial nucleotide sequence of the cDNA encoding protocadherin-PC (cloned from LNCaP-TR cells) and the corresponding amino acid sequence from the longest open reading frame within the cDNA. Underlined (bold) amino acid stretches identify cadherin-box motifs within the 5-region of the translation product. Crossed-through amino acids identify potential transmembrane domain of encoded peptide. However, translation product lacks signal sequence necessary to establish membrane localization.

Figure 2. Western blot analysis for the expression of protocadherin-PC protein in prostate cancer cell lines and tumor xenografts. A rabbit polyclonal antiserum made against a peptide of protocadherin-PC was used to probe a Western blot containing electrophoresed whole cell protein extracts from parental LNCaP cells, or the apoptosis-resistant derivatives LNCaP-TR and -SSR. As well, whole cell protein extracts made from LNCaP tumor xenografts maintained in intact male nude mouse (unlabeled, left lane), a xenograft at 2 days or 4 weeks after castration of the host mouse were electrophoresed and transferred to the Western blot. The antiserum recognized an approximately 110 kd protein (predicted size of protocadherin-PC peptide, 105.7 kd) that is highly expressed in apoptosis-resistant LNCaP variants and in LNCaP xenografts after castration in comparison to parental LNCaP cells or xenografts grown in androgenically-intact hosts.

Figure 3. Cell fractionation to identify subcellular localization of protocadherin-PC protein in prostate cancer cells. Separated cell fractions from LNCaP-TR or -SSR cells were electrophoresed on SDS-PAGE then transferred to a nitrocellulose filter to make a Western blot. The blot was probed with anti-Protocadherin-PC antiserum, revealing that the 110 kd protocadherin-PC protein is present in the cytoplasm of the apoptosis-resistant cell variants. This localization corresponds with the predicted inability of the protocadherin-PC protein to pass through the membrane (based on its lack of a signal sequence) from the amino acid sequence and is in contrast to the membrane localization of most other members of the cadherin gene family.

Figure 4. Binding of β -catenin protein to protocadherin-PC in vivo demonstrated by immunoprecipitation experiment. Immunoprecipitates made from different LNCaP cell variants were electrophoresed on SDS-PAGE and were transferred to a nitrocellulose filter. The Western blot was then probed with an antibody that recognizes human β -catenin. Results demonstrate that β -catenin protein is co-precipitated with protocadherin-PC in extracts from apoptosis-resistant cells, demonstrating the in vivo binding of these two proteins. Control lane in which purified recombinant human β -catenin protein was electrophoresed is shown in left.

Figure 5. Analysis of prostate cancer cell fractions for distribution of cadherin and catenin proteins. LNCaP cell variants were fractionated into membrane, cytosol and nuclear fractions. The proteins in these fractions were electrophoresed on SDS-PAGE and transferred to a nitrocellulose filter. The Western blots were probed with antibodies against human E-cadherin (top panels), α -catenin (a structural protein) (middle panel) or β -catenin (bottom panel). Results show that E-cadherin is entirely restricted to the membrane fraction of all cell variants as would be predicted; α -catenin is equally distributed between the membrane and cytoplasm of all cell variants and β -catenin, restricted to the membrane fraction of the parental LNCaP cells is now found extensively within the cytoplasmic and nuclear fractions of the LNCaP-TR and -SSR cells.

Figure 6. Expression of a luciferase reporter promoted by a β -catenin response element in prostate cancer cell variants. (A) Standardized luciferase activity when LNCaP cell variants were transiently transfected (48 hrs) with pTOP, an expression vector in which a luciferase reporter is promoted by multiple β -catenin promoter elements. Assay is standardized by comparison with activity of CMV-promoted β -galactosidase reporter transfected along with pTOP plasmid. (B) Standardized luciferase activity when LNCaP cell variants were transiently transfected (48 hrs) with pTOP compared with transient transfection with an equivalent of pFOP, modified from pTOP by inactivating mutations in the β -catenin promoter elements. Results demonstrate significantly enhanced transcriptional activation from the β -catenin/LEF-1 promoter elements in the apoptosis resistant LNCaP variants (-TR and -SSR) when compared to parental LNCaP cells.

Figure 7. Comparison of 5' transcribed sequence of protocadherin-like gene present on human X-chromosome to protocadherin gene present on human Y chromosome. Nucleotide sequence (top row) of transcript of X-chromosomal protocadherin-like gene and Y-chromosomal protocadherin gene are virtually similar except for a stretch of 13 bp (identified by small arrows) that is present in the X-chromosomal gene. The addition of this sequence within the X-chromosomal transcript permits the utilization of an alternate AUG site that results in a protocadherin-PC-like peptide containing a signal sequence and the potential for insertion into the membrane (unlike protocadherin-PC). The X-chromosomal gene is normally expressed in human brain and placenta whereas to our knowledge, the Y-chromosomal gene is only expressed in the human prostate gland.

Figure 8. Expression of protocadherin-PC gene in human prostate cancer specimens. a) semi-quantitative RT-PCR assay to quantify expression of protocadherin-PC in human prostate tumor RNA specimens. In this assay expression of protocadherin-PC mRNA (larger fragment) is compared to expression of TBP mRNA (a housekeeping transcription factor) (smaller fragment) in the LNCaP derivatives and in a series of normal human prostate epithelial or stromal cell lines. Results of assay (quantified by densitometry in lower graph) greatly enhanced expression of protocadherin-PC in apoptosis-resistant LNCaP variants and lack of expression of this gene product in normal prostate epithelial or stromal cell lines. b) Densitometric quantification of protocadherin-PC/TBP expression ratio in RNAs extracted from various human prostate tissues. Results show that normal human prostate tissues (derived from peripheral zone [PZ], central zone [CZ] or transitional zone [TZ] of prostate) express a similar, low level of protocadherin-PC. This expression is similar to that found in BPH or in untreated

prostate cancer specimens [UT]. In contrast, expression is significantly elevated in the hormone resistant [HR] prostate tumor specimens analyzed ($p < 0.01$). C) Modified RT-PCR assay that enables the identification of expression patterns of protocadherin-PC (from Y-chromosome) and protocadherin-PC-like homologue present on the X-chromosome in human prostate cells and tissues. This assay was performed using PCR primer sets that span the region of protocadherin-PC that differs (based on a 13 bp insert in the X-chromosomal gene). Results show that the X-chromosomal gene product (568 bp) is expressed at similar low levels in all prostate cells and tissues (normal and cancer) whereas the Y-chromosomal gene product is expressed at low levels in parental LNCaP cells, normal prostate and untreated prostate cancer and in much higher levels in the apoptosis-resistant variants of LNCaP and the hormone-resistant human prostate tumors.

871 ACTACTATGCAGTTCAAGCTAGTGTACAAGACCGGAGATGTGCCACTGATTGCAATTGAAGAGGATACTGGTGAGATCTTCACTACCGGCGCTCGCATTGATCGTGAGAAATTATGTGCT
1 M Q F K L V Y K T G D V P L I R I E E D T G E I F T T G A R I D R E K L C A

991 GGTATCCCAAGGGATGAGCATTGCTTTTATGAAGTGGAGTGGCCATTTTGCCGGATGAAATATTTAGACTGGTTAAGATACGTTTCTGATAGAAGATATAAATGATAATGCACCATG
39 G I P R D E H C F Y E V E V A I L P D E I F R L V K I R F L I E D I N D N A P L

1111 TTCCACGCAACAGTTATCAACATATCAATTCCAGAGAAGTCCGGCTATAAATCTAAATATACTCTCCACGCGGCTGTTGATCCTGACGTAGGCATAAACGGAGTTCAAACTACGAACTA
79 F P A T V I N I S I P E N S A I N S K Y T L P A A V D P D V G I N G V Q N Y E L

1231 ATTAAGAGTCAAAACATTTTGGCCTCGATGTCATTGAAACACCAGAAGGAGACAAGATGCCCAACTGATTGTTCAAAAGGAGTTAGATAGGGAAGAGAAGGATACCTATGTGATGAA
119 I K S Q N I F G L D V I E T P E G D K M P Q L I V Q K E L D R E E K D T Y V M K

1351 GTAAAGGTGAAGATGGTGGCTTCCCTCAAAGATCCAGTACTGCTATTTTGCAAGTAAAGTGTACTGATACAAATGACAACCCAGCTCTTTAAGGAGACAGAGATTGAAGTCAGTATA
159 V K V E D G G F P Q R S S T A I L Q V S V T T D T A N D N H P V F K E T E I E V S I

1471 CCAGAAAATGCTCCTGTAGGCACTTCAGTGACACAGTCCATGCCACAGATGCTGCATAGGTGAAATGCCAAGATCCACTTCTCTTTACGCAATCTAGTCTCCAACATGCCAGGAGA
199 P E N A P V G T S V T Q L H A T D A D I G E N A K I H F S F S N L V S N I A R R

1591 TTATTTACCTCAATGCCACCTGGACTTATCACAATCAAGAACCAGTGGATAGGGAAGAAACACCAACCAAGTTACTGGTTTGGCAAGTGTGTTGGATTGATGCCAGCAAGA
239 L F H L N A T T C T G L I T I K E P L D R E E T P N H K L L V L A S D G G L M P A R

1711 GCAATGGTGTGTAATGTACAGATGTCATGATAATGTCCCATCCATTGACATAAGATACATCGTCAATCCTGTCAATGACACAGTGTGTTCTTTAGAAAAATTTCCACTCAACAC
279 A M V L V N V T D V N D N V P S I D I R Y I V N P V N D T V V L S E N I P L N T

1831 AAAATGCTCTCATAACTGTGACGGATAAGATGCGGACCATAATGGCAGGTTGACATGCTTACAGATCATGAAATCCTTTAGATTAAAGGCCAGTATTAGTAATCAGTCTCTCTG
319 K I A L I T V T D K D A D H N G R V T C F T D H E I P F R L R P V F S N Q F L L

1951 GAGAATGCAGCATATCTTACTATGAGTGCACAAAGAAATATGCCATTAAATTAAGTGGTGCAGATGCTGGCAACCTCCTTTGAATCAGTCAGCAATGCTCTTCATCAAGTGAAGAT
359 E N A A Y L D Y E S T K E Y A I K L L A A D A G K P P L N Q S A M L F I K V K D

2071 GAAAATGACAATGCTCCAGTTTTCACCCAGTCTTTTCGTAATGTTTCTATCTCTGAGAATACTCTCTGGCATCCAGTTGATGAAAGTAAAGTGAACGGATGCAGACAGTGGGCTCAAT
399 E N D N A P V F T Q S F V T V S I P E N N S P G I Q L M K V S A T D A D S G P N

2191 GCTGAGATCAATTACCTGCTAGGCCCTGATGCTCCACCTGAATTCAGCCTGGATGCTGCTACAGGCATGCTGACTGTAGTGAAGAACTAGATAGAGAAAAAGAGGATAAATATTATTC
439 A E I N Y L L G P D A P P E F S L D R R T G M L T V V K K L D R E K E D K Y L F

2311 ACAATCTTGGCAAAAGATAATGGGGTACCAACCTTAACAGCAATGTACAGATCTTTGTAAGCATTATTGATCAGAATGACAATAGCCAGTTTCACTCACAATGAATACAAATCTAT
479 T I L A K D N G V P P L T S N V T V F V S I I D Q N D N S P V F T H N E Y K F Y

2431 GTCCACAGAAAACCTTCCAGGCAATGGTACAGTAGGACTAATCACTGTAATGATCCTGATTATGGAGACAATCTGCAGTTACGCTCTCCATTTAGATGAGAATGATGACTTCACCAT
519 V P E N L P R H G T V G L I T V T D P D Y G D N S A V T L S I L D E N D D F T I

2551 GATTCAAAAAGTGGTTCATCCGACCAAAATATTTCAATTTGATAGAGAAAAACAAGATCTTACACTTTCTATGTAAGGCTGAGGATGGTGGTAGAGTATCAGTCTCTCAAGTGCCAAA
559 D S Q T G V I R P N I S F D R E K Q E S Y T F Y V K A E D G G R V S R S S S A K

2671 GTAACCAATAAATGGTGTGATGTCATGACAACAACAGCTTTTCATTGTGCTCCTTACAATATTCTTATGAATGGTTTACCGTCCACTAATCCAGGCACAGTGGTCTTTAGGTA
599 V T I N V V D V N D N K P V F I V P P Y N Y S Y E L V L P S T N P G T V V F Q V

2791 ATTGCTTTGACAATGACACTGGCATGAATGCAGAGTTCGTTACAGCATTGTAGGAGGAAACACAAGAGATCTGTTTGCAATCGACCAAGAAACAGGCAACATAACATTGATGGAGAAA
639 I A V D N D T G M N A E V R Y S I V G G N T R D L F A I D Q E T G N I T L M E K

2911 TGTGATTTACAGACCTTGGTTACACAGATGTTGGTCAAGCTAATGACTTAGGACAGCCTGATTCTCTCTCAGTGTGTAATTGTCAATCTGTTGCTGAATGAGTCACTGACCAAT
679 C D V T D L G L H R V L V K A N D L G Q P D S L F S V I V N L F V N E S V T N

3031 GCTACAGTGAATTAATGAAGTGGTGGCAAAATGATTGAAGCAGCAGTACCCCAATACTGAGATAGCTGATGATCCTCACCACACTAGTGAATATGTCAAGATCTGGTTGACGCTGT
719 A T L I N E L V R K S I E A P V T P N T E I A D V S S P T S D Y V K T L V A A V

3151 GCTGGCAGCATAACTGCTGTTAGTTATTTCACTGCTGTAGTAAGATGTCCGACAGCACCACCTTAAGGCTGCTCAGAAAAACATGCAGAATCTGAATGGGCTACCCCAAC
759 A G T I T V V V V I F I T A V V R C R Q A P H L K A A Q K N M Q N S E W A T P N

3271 CCAGAAAACAGGCAGATGATAATGATGAAGAAAAAGAAAAAGAAAGAACATTCCCTTAAGAACTGCTGCTTAATTTGTCACTATTGAAGAACTAAGGCAGATGATGTTGACAGT
799 P E N R Q M I M K K K K K K H S P K N L L L N F V T I E E T K A D D V D S

3391 GATGGAACAGAGTCACACTAGACCTTCTATTGATCTAGAAGAGCAAAACATGGGAAAGTACAATTGGGTAACACACCTACTACTTTCAAGCCTGACAGCCTGATTGGCCCGACAC
839 D G N R V T L D L P I D L E E Q T M G K Y N W V T T P T T F K P D S P D L A R H

3511 YACAAATCTGCTCTCCACAGCCTGCCTTCCAAATTCAGCCTGAAACTCCCTGAATTTGAAGCACCACATCATCCAAGAACTGCCTCTCGATAACACCTTTTGGCCTGTGACTCTATC
879 K S A S P Q P A F Q I Q P E T P L N L K H H I I Q E L P L D N T F V A C D S I

3631 TCCAAGTGTCTCTCAAGCAGTTTCAGATCTCTACAGCTTTTCTGACTGTGGCTATCCAGTGACAACCTTCGAGGTACCTGTGTCCTGACACACAGACCGACTGATCCAGGACATGAAGT
919 S K C S S S S S S D P Y S V S D C G Y P V T T F E V P V S V H T R P T D S R T

FIGURE 1.

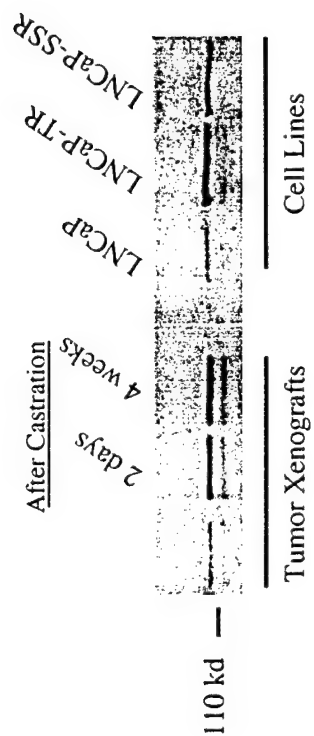


FIGURE 2

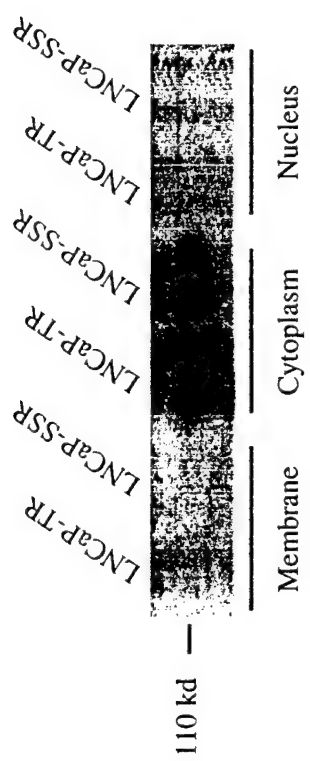


FIGURE 3

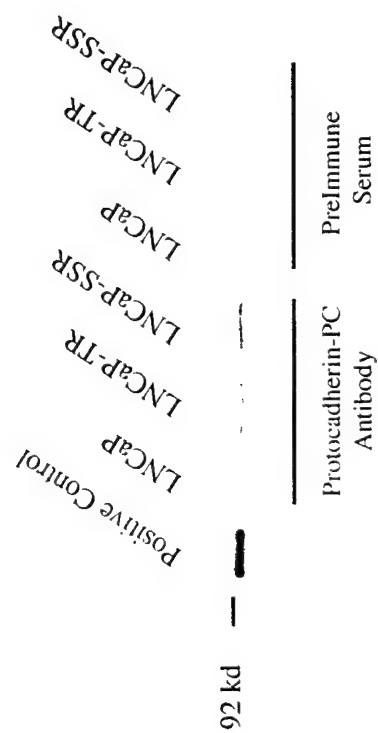


FIGURE 4

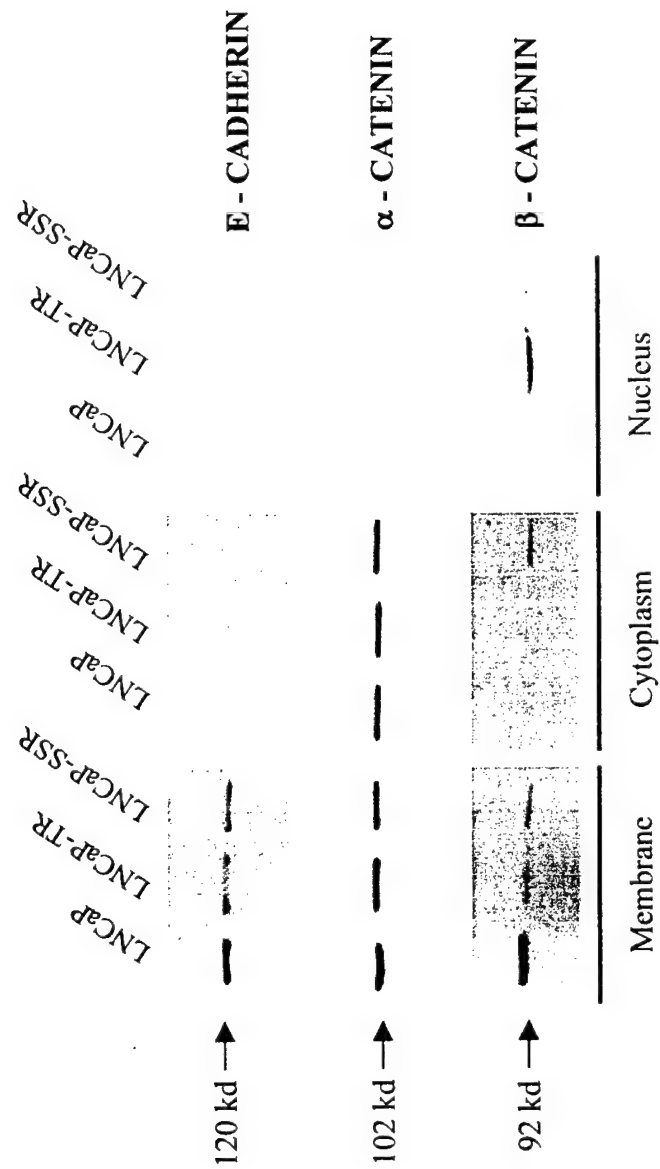


FIGURE 5

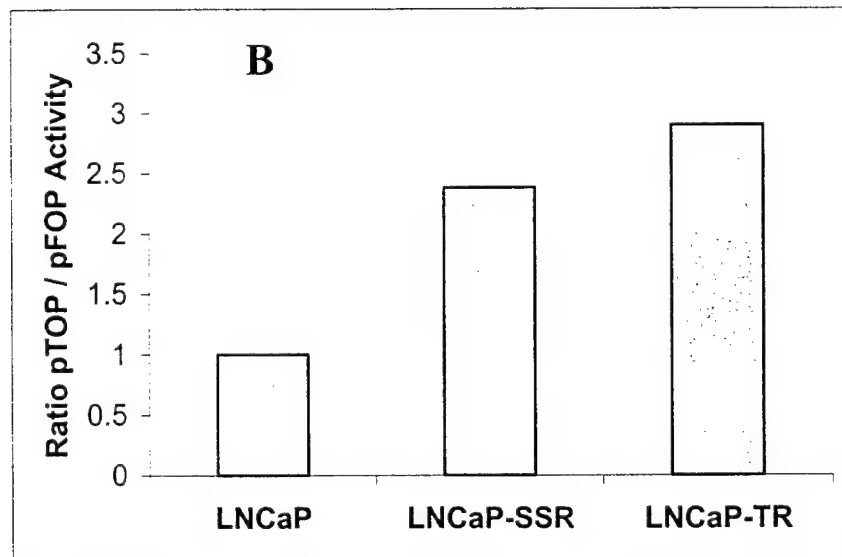
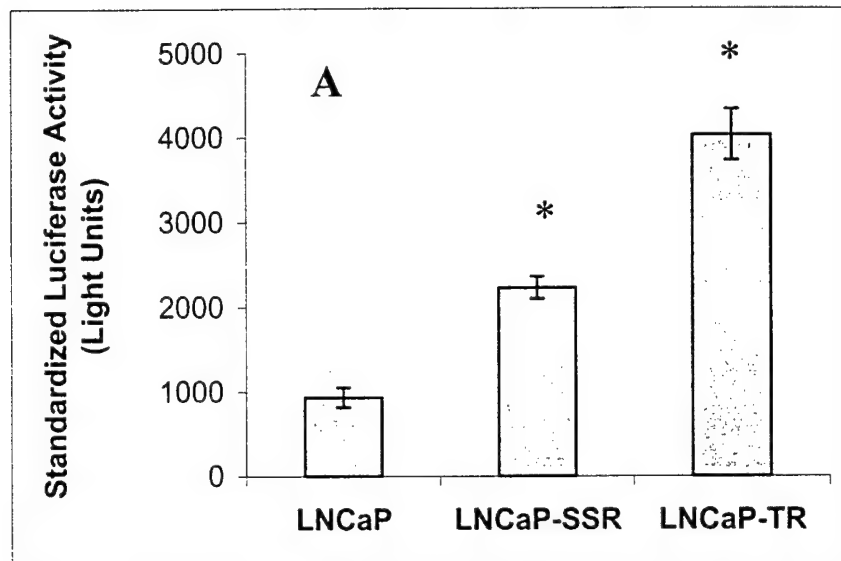


FIGURE 6

601 CTATGAGGACTGAACGACAGTGGGTTTAAATTCAGATAATTTCAAGTGTGTCGGGTTAATAACAACTTG
M R T E R Q W' V L I Q I F Q V' L C G L I Q Q T V
* T T V G F N S D I S S V V R V N T T N C + 13 bp sequence
- 13 bp sequence

↓

TCACAAAGTGTACCTGGTATGGACTTGTGTCGGGACGTACATTTTCGGGTCCTGCTAGTATGCGTGGTGTTTC
T S V P G M D L L S G T Y I F A V' L L V' C V' F
H K C L L S G T Y I F A V L L V C V V F + 13 bp sequence
- 13 bp sequence

▼

CACTCTGGCGCCAGGAGAAAAAACTACACCATCCGAGAGAAATTCAGAAAAAGTCTGTATAGGCAACTTG
H S G A Q E K N Y T I R E E I P E N V' L I G N L + 13 bp sequence
H S G A Q E K N Y T I R E E I P E N V L I G N L - 13 bp sequence

TTGAAAAGACCTTAACCTTGTGCTGATTCCAAACAAGTCTTGACAACTACTATGCAGTTCAAAGCTAGTG....
L K D L N L S L I P N K S L T T T M Q F K L V..... + 13 bp sequence
L K D L N L S L I P N K S L T T T M Q F K L V..... - 13 bp sequence

FIGURE 7

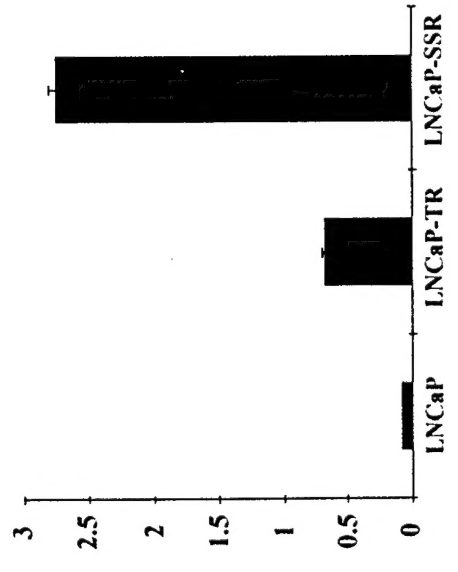
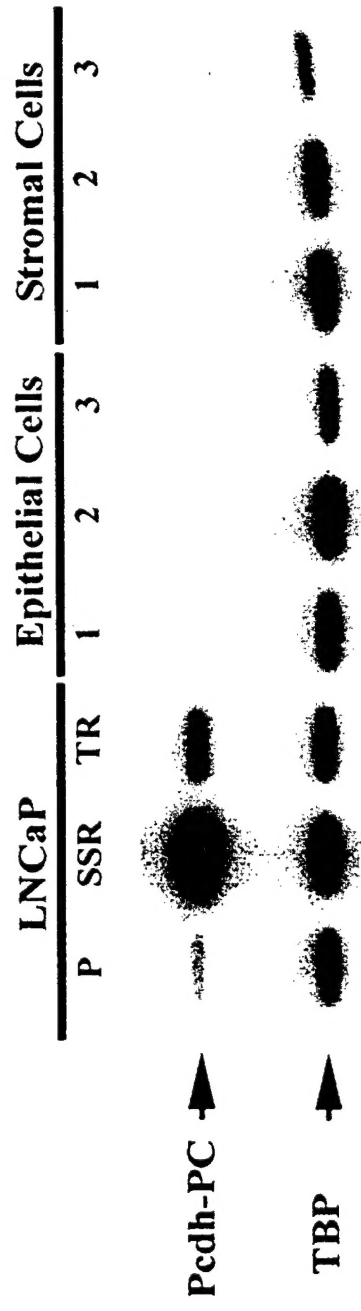


FIGURE 8A

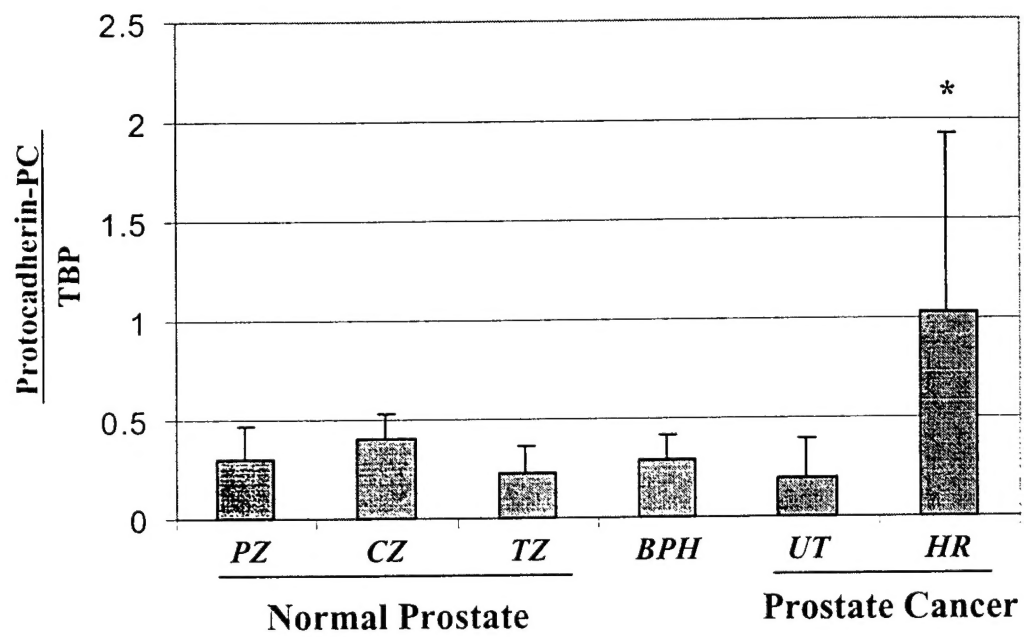


FIGURE 8B

Pcdh-PC isoforms

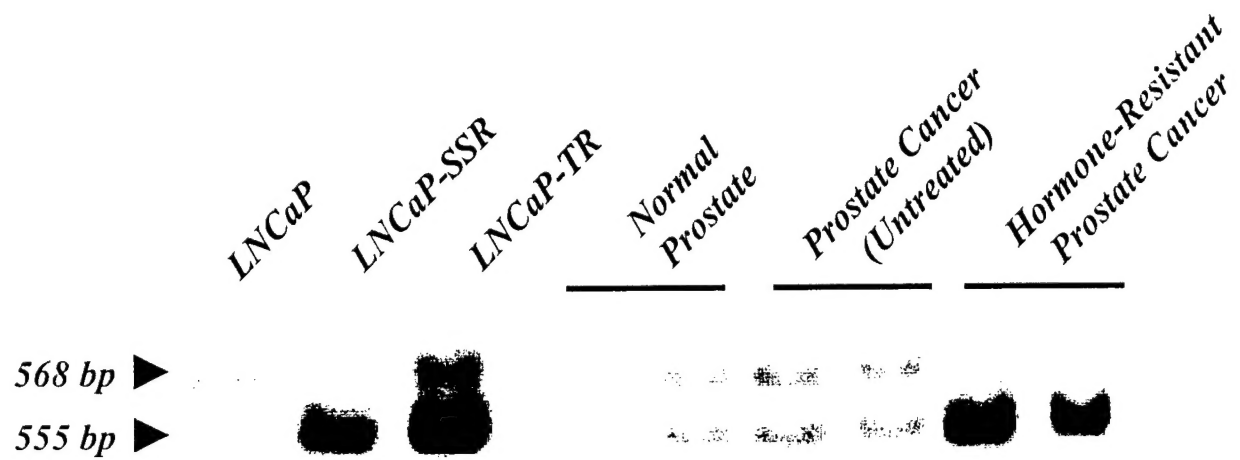


FIGURE 8C